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Effect of passage through the plant on virulence and endophytic behavioural adaptation in the entomopathogenic fungus *Beauveria bassiana*

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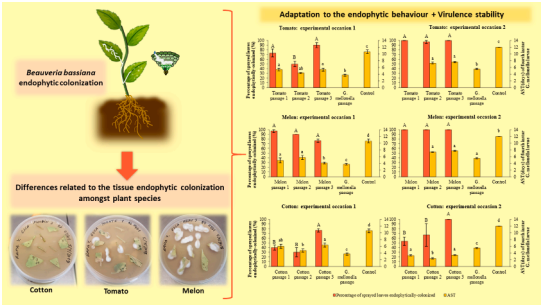
HIGHLIGHTS

- *B. bassiana* endophytically colonized melon, tomato and cotton plants.
- Cotton was the the least suitable host plant for endophytic colonization.
- *B. bassiana* re-isolation rate from cotton plants increased after the first passage.
- This is the first report an of improvement of endophytic capacity by passaging.
- Virulence stability after successive passage through a host plant was demonstrated.

GRAPHICAL ABSTRACT



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ABSTRACT

Systemic crop protection using endophytic isolates of entomopathogenic fungi is at the forefront of IPM. Nonetheless, any potential trade-offs between virulence and endophytic behaviour must be elucidated if they are to be effectively used in pest management strategies. Here we investigated endophytic adaptation in an isolate of *Beauveria bassiana* following successive passage through melon, tomato and cotton tissues. Plants were sprayed with a suspension of *B. bassiana* endophytic isolate EABb 04/01-Tip to initiate endophytic colonization. Once colonization was established, the fungus was re-isolated from the plant, applied to another plant and re-isolated again; this was repeated to achieve three passages. After each passage, a conidial suspension of each isolate was used in bioassays to evaluate both virulence against 4th instar larvae of the model insect *Galleria mellonella* and to quantify the extent of endophytic activity in each respective host plant species. When sprayed leaves were inspected for fungal colonization, differences in percentage tissue colonization amongst the plant species were detected after the first re-isolation. Endophytic colonization rates in melon and tomato, which varied from 70 to 100%, were higher than those observed in cotton, which ranged from 40 to 50%; endophytic colonization in cotton increased to 75–100% after the third passage. This improvement in endophytic behaviour in cotton, an apparently suboptimal plant for fungal colonization, suggests an evolutionary adaptation to localized or transient endophytic colonization, while further assays are needed. Meanwhile, when endophytic colonization of non-sprayed leaves distant from the sprayed ones was investigated, endophytic activity was evident in all three crop species suggesting that movement within plants after successive passage increased the extent of endophytic colonization from transient to systemic. The present research highlights the potential for adaptation to

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endophytic behaviour in crops that are less suitable for endophytic colonization. Furthermore, we demonstrated stability in virulence after multiple passages through host plants. This is a key result for the development of IPM strategies based on endophytic entomopathogenic fungi.

1. Introduction

Entomopathogenic fungi are traditionally considered as a large group of species that naturally infect and regulate insect and mite populations, and that their main habitats are soil and insect cadavers (Lovett and St. Leger, 2017). Recently it has been shown that isolates of some entomopathogenic fungi exhibit additional and complementary ecological functions, establishing plant associations in the phylloplane, rhizosphere and as endophytes (Hu and Bidochka, 2021; Meyling and Eilenberg, 2006; Quesada-Moraga, 2020; Vega et al., 2008). Through the endophytic pathway, they can systemically protect the plant against chewing and sap-sucking pests (Garrido-Jurado et al., 2017; González-Mas et al., 2019; Quesada-Moraga et al., 2009; Resquín-Romero et al., 2016). Endophytic entomopathogenic fungi have also been shown to protect plants against phytopathogenic microorganisms and plant viruses, enhance responses to abiotic stresses, promote plant growth, and attract natural enemies (Quesada-Moraga, 2020). All of these features make these biological control agents very desirable for use in integrated pest management (IPM) systems. It has been suggested that entomopathogenic fungi evolved from species that were plant associates but subsequently gained the ability to infect and kill insects (Barelli et al., 2016; Gao et al., 2011; Stone and Bidochka, 2020); amongst these, some entomopathogenic fungi retained their ancestral ability to also colonize various plant species. However, not all entomopathogenic fungi have this dual lifestyle and the degree of plant tissue colonization can vary depending on host plant species (Gurulingappa et al., 2010; Resquín-Romero et al., 2016; Vega, 2018). The entomopathogenic fungal species most frequently showing endophytic behaviour are found in the genera *Beauveria* and *Metarhizium* (Ascomycota: Hypocreales), which typically have broad insect host ranges and can endophytically colonize many important crop species (Vega, 2018). These species and isolates with dual lifestyles have been inoculated on to several important crops for artificial establishment (Moonjely and Bidochka, 2019; Vega, 2018). However, it is not known whether the opposite process is possible, i.e. that strict entomopathogenic fungal isolates can adapt to an endophytic lifestyle, or that the endophytic capacity of isolates can be improved through adaptation in target host plant species.

While there is strong evidence for lethal and sublethal effects on insects of feeding on endophytically-colonized plants, signs of fungal outgrowth on the cadavers are rare, meaning the definitive cause of the mortality remains elusive (Garrido-Jurado et al., 2017; Resquín-Romero et al., 2016; Sánchez-Rodríguez et al., 2018). It has been proposed that these negative effects on herbivorous insects could be as a result of antibiosis and feeding deterrence mediated by *in planta* production of fungal and plant secondary metabolites as a consequence of plant defense induction (Akello et al., 2008a; Cherry et al., 2004; Vega, 2018). Nevertheless, several studies have reported fungal outgrowth on insects that died after feeding on plants endophytically-colonized by the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuill. (Ascomycota: Hypocreales) (Akello et al., 2008b, 2008a; Garrido-Jurado et al., 2017; Jaber and Vidal, 2010; Klieber and Reineke, 2016; Powell et al.., 2009; Powell et al., 2007).

It has been proposed that genes involved in virulence against insects may have been co-opted from genes involved in plant colonization or from horizontal gene transfer during fungal evolution (Screen and St. Leger, 2000). This adaptation may involve penetration-related genes that regulate penetration of the insect cuticle (from both the inside and the outside) and so affect conidia production on the host (Zhang et al., 2010). It is still not understood whether these endophytic entomopathogenic fungi are less evolved than strict entomopathogens, or whether they have just followed a different evolutionary pathway in which both pathogenic and endophytic colonization genes are maintained, while virulence-related genes are inactivated during plant colonization. Furthermore, whether entomopathogenic fungi lose pathogenic capacity when adapting to grow in non-insect habitats, as occurs when they are cultivated in artificial medium, is poorly understood (Ansari and Butt, 2011). Stability of insect virulence in endophytic entomopathogenic fungi is a key goal of present and future research.

Thus, we studied endophytic adaptation in *B. bassiana* EABb 04/01-Tip, an isolate from a cynipid borer larva with known endophytic capacity. We focused on adaptation after serial passage *in planta* through three crop species (melon, tomato, and cotton), and whether endophytic capacity was increased. Stability of virulence after plant passage was also evaluated.

2. Materials and methods

2.1. Biological material: plants, fungal isolate and insect populations

For experiments, melon (*Cucumis melo* L. var. Galia Híbrido F1), tomato (*Solanum lycopersicum* L. cv. Tres cantos) and cotton (*Gossypium hirsutum* L. cv Elsa) seeds were surface sterilized in 2% NaOCl (Sigma-Aldrich, MO, USA) for 2 min, and rinsed twice with sterile Mili-Q water under sterile laminar-flow conditions. The substrate in which they were to be grown was sterilized twice in an autoclave for 20 min at 121 °C with a 24-h interval between each sterilization process. Surface-sterilized seeds were germinated in 9x9 cm pots in a mixture of equal parts of sterilized vermiculite (No. 3, Asfaltex S.A., Barcelona, Spain) and soil substrate (Floragard, Oldenburg, Germany) and maintained in an environmental chamber under controlled conditions: 25 ± 2 °C, 16: 8 h light: dark regime. Plants were watered three times a week and a nutrient complex of 20: 20: 20 (N: P: K) Nutrichem 60 fertilizer (Miller Chemical & Fertilizer Corp., Hanover, PA, USA) added to the irrigation water at a rate of 1 g/l.

Beauveria bassiana EABb 04/01-Tip 04/01-Tip was the isolate used in this study which originated from a larva of the opium poppy stem gall, *Iraella luteipes* (Thompson) (Hymenoptera: Cynipidae), found in an opium poppy crop in Ecija (Sevilla, Spain). The isolate was deposited in the University of Córdoba Entomopathogenic Fungi Collection, Córdoba, Spain and in the Spanish Collection of Culture Types (CECT), University of Valencia (accession n° CECT 20744). Nucleotide sequences for the ITS of EABb 04/01-Tip can be found in the Gen-Bank database (FJ972963). The endophytic activity of this isolate in opium poppy, melon and tomato plants had been demonstrated previously as has its ability to protect these crops from sap-sucking and chewing pests (Garrido-Jurado et al., 2017; González-Mas et al., 2019; Quesada-Moraga et al., 2009; Resquín-Romero et al., 2016). For all bioassays, a monosporic isolate of *B. bassiana* EABb 04/01-Tip was grown over cellophane film on potato dextrose agar (PDA) (Becton Dickinson Franklin Lakes, NJ, USA) in Petri dishes; the cellophane between the agar and the fungus was used to prevent nutrients entering the conidial suspensions at harvest. Cultures were incubated for 15 days at 25 °C in darkness and conidial suspensions were prepared by scraping the fungus from the cellophane into a sterile aqueous solution of 0.01% Tween 80. The resulting suspension was filtered through several layers of sterile cheesecloth to remove mycelia and sonicated for 5 min to homogenize the inoculum. The concentration of conidia used for inoculation was determined using a haemocytometer and appropriate dilutions were made in 0.01% Tween 80 to obtain the selected conidial concentration. Prior to experimentation, conidial viability was determined on liquid

Czapek-Dox broth plus 1% (w/v) yeast extract medium and only suspensions with > 97.0% germination after 24 h, were used.

The greater wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae) was selected as a model host in these experiments because it has been used widely to compare the virulence of entomopathogenic fungi (Fuchs et al., 2010). Larvae were obtained from a healthy colony established at the Department of Agronomy of the University of Córdoba (Spain) that had been reared following the method of Dutky et al., (1962). Briefly, larvae were reared on an artificial diet consisting of a mixture of 30.8 g of corn flour, 30.8 g of wheat germ, 30.8 g of wheat bran and 10.8 g of brewer's yeast, with 27 ml of glycerol and 48.6 ml of honey per 200 g of artificial diet. They were maintained in an environmental chamber programmed at 26 ± 2 °C and 70 ± 5% RH, with a photoperiod of 16: 8 (light: dark) h. For each experiment, 4th instar larvae were collected from the rearing cages and used immediately.

2.2. Inoculation of plants with entomopathogenic fungi, verification of endophytic colonization, and re-isolation of the fungus

In order to study possible adaptation to endophytic behaviour, *B. bassiana* EABb 04/01-Tip was successively recovered from tissues of crops from different botanical families. For each plant species, melon, tomato and cotton, three four-leaf-stage plants were treated (n = 3 per treatment). The leaves of each plant were sprayed with 2 ml of fungal suspension (10^8 conidia/ml.) using an aerograph 27,085 (piston compressor of 23 l/min, 15–50 PSI and a 0.3 mm nozzle diameter, China). After inoculation, all plants were covered by another plastic sheet to promote fungal growth for 24 h and incubated at 26 ± 2 °C and 70 ± 5% RH, with a photoperiod of 16: 8 (light: dark) h. Control plants were treated in the same way but only sprayed with sterile water with 0.01% Tween 80.

Plants were inspected for possible endophytic fungal colonization 72 h after spraying using methods we have described previously (Quesada-Moraga et al., 2009; Resquín-Romero et al., 2016; Garrido-Jurado et al., 2017; González-Mas et al., 2019). To confirm endophytic colonization and re-isolate the fungus, samples of leaves were collected from each replicate treatment and control plant (one per plant, n = 3 per treatment and plant species), surface-sterilized in 1% NaOCl for 2 min, rinsed twice in sterile distilled water and dried on sterile filter paper. Ten sections of approximately 2 cm^2 were cut from each leaf using a sterile scalpel and plated out individually in Petri dishes containing selective culture medium to determine the proportion of sections per leaf colonized endophytically; the medium contained: 20 g of Agar Sabouraud Glucose Chloramphenicol (Cultimed Panreac, Spain), 500 mg l-1 streptomycin sulfate (Sigma-Aldrich Chemie, China), 500 mg l-1 ampicillin (Intron Biotechnology, China) and 500 mg l-1 dodine 65 WP (Barcelona, Spain). We also plated out the last rinse water from each leaf separately to confirm the effectiveness of the surface-sterilization procedure as demonstrated by the absence of entomopathogenic fungal growth in these plates. All plates were incubated at 25 °C in darkness until fungal growth was observed; the absence of fungal growth in the controls confirmed that only the inoculated isolates were involved. After isolation, in order to cover the possible existence of different traits in the three plants from each plant-treatment combination, a pool including 2 isolates per plant (per leaf) for each plant species was deployed (therefore including 6 isolates, 2 each plant). Hence, considering that there were three plants per treatment, with one leaf per plant used for re-isolation of which ten leaf section were cut, two randomly selected isolates from each leaf were pooled with a total of 6 isolates from each treatment (two each plant). Recovered isolates were grown on PDA medium as described previously and a conidial suspension produced for inoculation of new plants (n = 3) of the same crop type. Percentage endophytic colonization was estimated from the proportion of sections from each leaf from which emerging fungus was observed. This procedure was repeated twice, i.e. growth re-isolation on PDA and colonization of new batches of plants; in total there were three passages for each crop type.

2.3. Virulence of passaged B. Bassiana isolates against fourth instar G. Mellonella larvae

In the present manuscript, use of the term virulence is based on the definition of Shapiro-Ilan et al. 2005. After each passage, recovered isolates were grown on PDA medium as described previously and a conidial suspension of each prepared and adjusted to a final concentration of 10^7 conidia/ml as described in 2.1. Conidial suspensions of all recovered isolates were assayed to estimate their virulence against 4th instar larvae of the model insect *G. mellonella*. In summary, nine recovered isolates (three successively isolated (one per passage) from melon plants, three from tomato and three from cotton) were compared with a negative control (sterile water with 0.01% Tween 80) and a positive control (conidial suspension of *B. bassiana* EABb 04/01-Tip recovered directly from a *G. melonella* cadaver). For assay, groups of *G. mellonella* larvae (ten larvae per group; three replicate groups per treatment/ control) were immersed directly for 30 s in an aqueous suspension of 10^7 conidia/ml or the control aqueous solution. After treatment, each group of larvae were transferred into Petri dishes (90 mm in diameter) and incubated at 25 °C. After 24 h without food, artificial diet was provided *ad libitum* and incubation continued at 25 °C for 12 further days. Mortality was recorded daily. Dead larvae were processed as follows to determine whether mortality was due to fungal infection: cadavers were individually surface disinfected in a solution of 1% sodium hypochlorite (2 min) and rinsed twice in distilled sterile water (1 min) to remove sodium hypochlorite residues. Subsequently, they were transferred individually to Petri dishes on moistened filter paper, sealed with Parafilm, and incubated in complete darkness at 25 ± 2 °C to stimulate fungal growth. In total, 330 larvae were used in this experiment.

The entire experiment (i.e. evaluating leaf colonization and isolate virulence) was done on two occasions, each time using fresh fungal inoculum and larvae. However, on the second experiment, two leaves on each replicate treatment plant were covered with a transparent plastic sheet prior to spraying to prevent them from being inoculated. Endophytic colonization of these leaves (one per plant) was evaluated in the same way as sprayed leaves to determine whether there had been within-plant movement of the fungus from sprayed leaves.

2.4. Statistical analysis

Data on the percentages of leaf fragments endophytically colonized by *B. bassiana* EABb 04/01-Tip were analyzed using Shapiro–Wilk and Levene's tests to calculate linear model assumptions (normality and homogeneity of variance). If the distribution was not normal data were transformed using an angular transformation (Steel and Torrie, 1985) prior to analysis. Comparisons amongst treatments were made using analysis of variance (ANOVA) (for Gaussian variables) or the Kruskal–Wallis nonparametric test (for non-Gaussian variables). Average survival times (ASTs) and cumulative survival ratios were obtained via Kaplan–Meier survivorship analysis (Kaplan and Meier, 1958) and compared via the log-rank test calculated. All analyses were done using IBM SPSS 25.0 software.

3. Results

3.1. Endophytic behaviour of B. Bassiana EABb 04/01-Tip after successive passage through melon, tomato or cotton

Microbiological techniques confirmed that *B. bassiana* EABb 04/01-Tip was recovered from all passages through the three selected crop species, i.e. endophytic behaviour was observed in all the replicate plants from all plant species 72 h after spraying. Despite that, amongst the different host plant species on each passage, there were differences

in the percentage of fragments of sprayed leaves from which the fungus was recovered: $F_{2,8} = 19.91$, $P = 0.002$ and $H_{2,8} = 7.62$, $P = 0.0001$ for the first passage on the experiment 1 and 2, respectively; $F_{2,8} = 21.00$, $P = 0.002$ and $H_{2,8} = 3.23$, $P = 0.211$ for the second passage on the experiment 1 and 2, respectively; $F_{2,8} = 3.20$, $P = 0.113$ for the third passage on the experiment 1. For the third passage on the experiment 2, all leaves were colonized at 100%. (Fig. 1). Overall, the percentage of leaves colonized was lower in cotton plants than in tomato and melon plants; on the first and second experiments, respectively, percentage colonization was 73.0 ± 8.8% and 100.0 ± 0.0% in tomato, 97.0 ± 3.3% and 100.0 ± 0.0% in melon and 40.0 ± 5.8% and 53.3 ± 8.8% in cotton after the first passage. However, these differences were less noticeable after the second passage through each plant, with a substantial increase in the percentage of colonized leaves in cotton plants, which increased to 76.7 ± 3.3% after the second passage and 100.0 ± 0.0% in the third passage. Passage also had a significant overall effect on the percentage of leaves colonized ($F_{2,8} = 8.38$, $P = 0.018$ and $H_{2,8} = 2$, $P = 0.422$ for tomato on the experiment 1 and 2, respectively; $F_{2,8} = 14.00$, $P = 0.006$ for melon on the experiment 1 with 100% of the leaves colonized on the experiment 2; $F_{2,8} = 12.54$, $P = 0.007$ and $F_{2,8} = 2.64$, $P = 0.150$ for

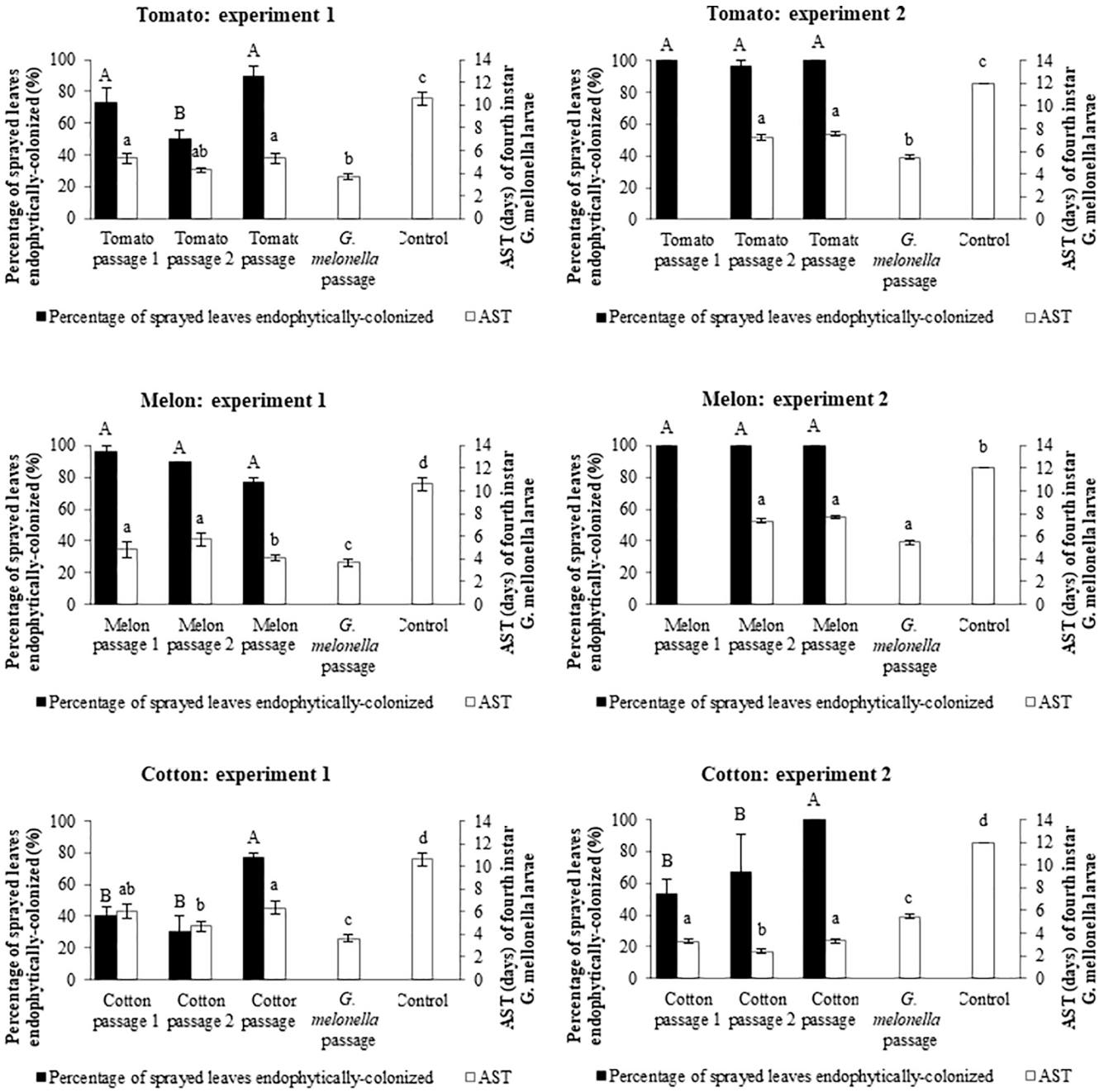

Fig. 1. Percentage of sprayed tomato, melon and cotton leaves endophytically colonized following foliar application of *B. bassiana* EABb 04/01-Tip that had been reisolated after different numbers of passages through each crop type and the virulence of each re-isolation to *Galleria mellonella*. Leaf colonization data (black bars) are presented as percentage means ± SE of fungus-positive fragments from sprayed leaves. Concentration of conidia applied was 1.0×10^8 conidia/ml on both experiments. Leaves were collected 72 h after inoculation. Means with the same uppercase letter are not significantly different to each other according to the LSD test ($p < 0.05$). Susceptibility data (white bars) were determined against 4th instar larvae at a concentration of 1.0×10^7 conidia/ml and presented as the average survival time (AST) ± SE. Means with the same lowercase letter in each crop are not significantly different to each other according to the log rank test ($p < 0.05$). Average survival time was limited at 12 days.

cotton on the experiment 1 and 2, respectively). *Beauveria bassiana* EABb 04/01-Tip achieved the highest percentage colonization of leaves in all crop plants when using inoculum from the second passage (Fig. 1).

On the second experiment, the endophytic colonization of unsprayed leaves was also evaluated and there was a significant effect of passage on fungal recovery rates from each plant species: $F = 3.43$, $P = 0.1015$ and $F = 9.71$, $P = 0.0132$ for passages 1 and 2 respectively, with 100% of the leaves being colonized following the third passage. The effect of passage on movement of fungal inoculum though the plant resulting in recovery from non-sprayed leaves was even more evident than for recovery from sprayed leaves; there was a steady increase across the three successive passages ($H_{2,8} = 7.72$, $P = 0.000$; $F_{2,8} = 3.91$, $P = 0.082$ and $F_{2,8} = 9.98P = 0.012$ for tomato, melon and cotton, respectively). For unsprayed leaves 73.3 ± 3.3%, 56.7 ± 0.0% and 23.3 ± 14.5% of leaves were colonized after the first passage, in tomato, melon and cotton plants, respectively; colonization rates reached 100.0 ± 0.0% in all plant types following the third passage. As for sprayed leaves, the endophytic capacity of the fungus improved after the second passage through cotton plants. However, in unsprayed leaves this increase was also observed in tomato and melon plants (Fig. 2). Thus, endophytic capability is dependent not only on the host plant species, but also on the number of passages through that plant type.

3.2. Effect of successive re-isolation from different plant species on virulence of B. Bassiana EABb 04/01-Tip

All the re-isolates of *B. bassiana* EABb 04/01-Tip were virulent against fourth instar larvae of *G. mellonella.* Signs of fungal penetration through the larval cuticle and natural insect openings were observed 2–3 days after treatment. When the larvae died, their colour changed from sandy to dark brown and this was followed by outgrowth of *B. bassiana* on the insect surface. This effect was enhanced by incubation of dead larvae for 7–10 days under optimal temperature and relative humidity conditions, which promoted fungal growth. It should be noted that all dead larvae that had been treated with *B. bassiana* conidial suspensions developed fungal outgrowth, while it was not detected in those dead larvae from the control treatment.

All isolates were highly virulent against *G. mellonella* larvae, resulting in significant mortality and a reduction in the AST. Total mortality of *B. bassiana*-treated larvae ranged from 73.3 to 100.0%, on the first experiment while on the second experiment it was 100.0 ± 0.0% in all treatments. No significant differences amongst isolates from the three successive passages were detected either in total mortality (all cadavers showing fungal outgrowth) nor in AST (Fig. 1). In contrast, significant differences were detected when comparing these isolates with the one obtained from passage through *G. mellonella*, which had the shortest AST values (3.7 ± 0.3 and 5.5 ± 0.2 on the first and second experiment, respectively. Thus, virulence of *B. bassiana* EABb 04/01-Tip did not change following repeated passage through each crop species.

4. Discussion

Overall, *B. bassiana* EABb 04/01-Tip achieved successful endophytic colonization of the three crop species evaluated (melon, tomato, and cotton), with high percentages of leaf fragments colonized 72 h after being sprayed. Previous studies have reported similar levels of endophytic colonization of these host plants by *B. bassiana* (Gurulingappa et al., 2010; Lopez and Sword, 2015; Resquín-Romero et al., 2016). However, of the three species compared, cotton plants were the least suitable for endophytic colonization compared with the other two species. This could be due to the presence of secondary plant compounds commonly produced by cotton plants, which are known to have antifungal properties, e.g. the terpenoid, gossypol, which is produced by many cotton cultivars (Benbouza et al., 2009; Egbuta et al., 2017; Mellon et al., 2012; Przybylski et al., 2009). This is in accordance with previous studies showing that several pest populations reared on cotton were significantly less susceptible to infection by the entomopathogenic fungi *Isaria fumosorosea* Wize (formerly *Paecilomyces fumosoroseus*) and *B. bassiana* than when reared on other host plants (Poprawski and Jones, 2000; Santiago-Álvarez et al., 2006). Such studies demonstrated that mortality caused by entomopathogenic fungi, and subsequent sporulation, was significantly influenced by the host plant on which the insects were feeding. In cotton, mean mortality of whitefly nymphs was reduced and ASTs lengthened compared with those reared on other plant species (Santiago-Álvarez et al., 2006). Furthermore, conidia production was reduced in nymphs reared on cotton compared with nymphs reared on other host plants. Conidia production was also significantly reduced in cadavers of hosts reared on host plants less favorable for the fungus (Santiago-Álvarez et al., 2006). These authors hypothesized that the cotton plant produced a fungal inhibitor that conferred protection against entomopathogenic fungi in insects feeding upon it (Poprawski and Jones, 2000; Santiago-Álvarez et al., 2006). Indeed, *in vitro* studies demonstrated that gossypol was likely to be involved in this antibiosis (Poprawski and Jones, 2000). Thus, a possibility is that some fungal inhibitors could infer in the endophytic colonzation of the plant, however further research is necessary to determinate that.

Although cotton was the poorer host plant, we observed that *B. bassiana* re-isolation rate from cotton plants increased after the first passage. Thus, it seems that passage through a less suitable host plant could improve endophytic activity in that plant and achieve higher colonization rates. This is the first time that evidence for improvement of endophytic capacity by passaging has been reported. The current research has two key outcomes. Firstly, when sprayed leaves were inspected for fungal colonization, prior passage improved endophytic behaviour in cotton, an apparently suboptimal plant for fungal colonization; this suggests an evolutionary adaptation to localized or transient

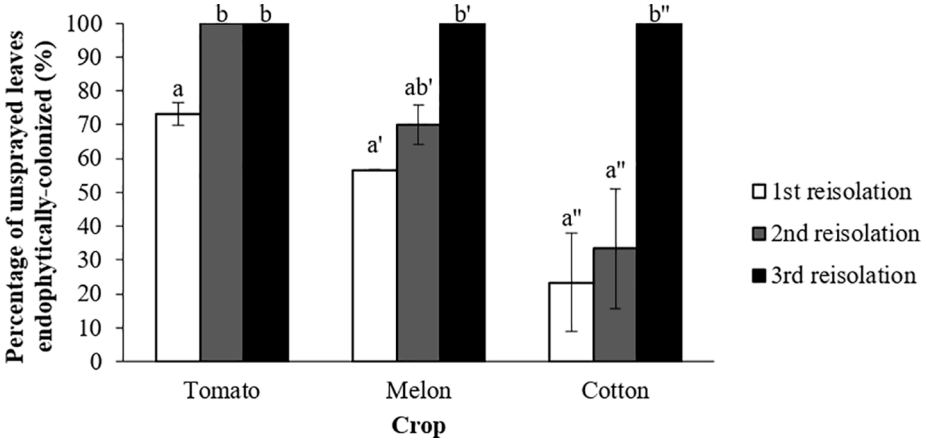

Fig. 2. Percentage of unsprayed tomato, melon and cotton leaves endophytically colonized following foliar application of *B. bassiana* EABb 04/01-Tip that had been reisolated after different numbers of passages through each crop type. Leaf colonization data are presented as means ± SE of fungus-positive fragments from unsprayed leaves. Concentration of conidia applied was 1.0×10^8 conidia/ml on both experiments. Leaves were collected 72 h after inoculation. Means with the same lowercase letter are not significantly different to each other according to the LSD test ($p < 0.05$).

endophytic colonization. Furthermore, when unsprayed leaves were inspected for fungal colonization, endophytic behaviour (as measured by within-plant movement) of isolates was also improved by passage in all three crops suggesting that passage through a plant host could improve the extend of endophytic colonization from transient to systemic, and thus achieve higher colonization rates.

This research is the first report of stability in virulence of entomopathogenic fungi after successive passage through a host plant. All isolates achieved similar mortality and ASTs after three passages through the plant; as expected, passage through an insect host improved virulence (Aizawa, 1971; Butt and Goettel, 2000; Quesada-Moraga and Vey, 2003). As passage through the plant neither increased nor decreased fungal virulence we suggest that endophytic colonization stabilized virulence; this is a key result for the development of IPM strategies incorporating endophytic entomopathogenic fungi. However, virulence stability during passage could be isolate dependent, with some isolates conserving virulence while others become attenuated after serial passage, as occurs after multiple subcultures on artificial medium (Butt et al., 2006; Loesch et al., 2010; Shah and Butt, 2005). Thus, identification of an isolate that retains stable virulence after a period of endophytic growth is an important selection criterion for IPM. Nonetheless, the reported occurrence of natural *in planta* insect infection cycles could enable continuous virulence restoration (Garrido-Jurado et al., 2017; Keyser et al., 2014; Resquín-Romero et al., 2016; Rivas-Franco et al., 2020).

In summary, our results support the hypothesis that passage through a host plant can improve endophytic activity and achieve higher colonization rates. Future research on the genes involved in endophytic activity may help identify the mechanisms of this adaptation. This finding could improve fungal host plant specificity for use in IPM. This research also demonstrated virulence stability in isolates following repeated passage through three different plant species. Thus, the use of endophytic entomopathogenic fungi for control of insect pests can be tailored to melon, tomato and cotton, with no loss of virulence.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Aizawa, K., 1971. Strain improvement and preservation of virulence. In: Burges, H., Hussey, N. (Eds.), Microbial Control of Insects and Mites. Acad. Press, New York, pp. 655–672.

Akello, J., Dubois, T., Coyne, D., Kyamanywa, S., 2008a. Effect of endophytic Beauveria bassiana on populations of the banana weevil, Cosmopolites sordidus, and their damage in tissue-cultured banana plants. Entomol. Exp. Appl. 129, 157–165. https://doi.org/10.1111/j.1570-7458.2008.00759.x.

Akello, J., Dubois, T., Coyne, D., Kyamanywa, S., 2008b. Endophytic Beauveria bassiana in banana (Musa spp.) reduces banana weevil (Cosmopolites sordidus) fitness and damage. Crop Prot. 27 (11), 1437–1441. https://doi.org/10.1016/j.cropro.2008.07.003.

Ansari, M.A., Butt, T.M., 2011. Effects of successive subculturing on stability, virulence, conidial yield, germination and shelf-life of entomopathogenic fungi. J. Appl. Microbiol. 110, 1460–1469. https://doi.org/10.1111/j.1365-2672.2011.04994.x.

Barelli, L., Moonjely, S., Behie, S.W., Bidochka, M.J., 2016. Fungi with multifunctional lifestyles: endophytic insect pathogenic fungi. Plant Mol. Biol. 90 (6), 657–664. https://doi.org/10.1007/s11103-015-0413-z.

Benbouza, H., Lognay, G., Scheffler, J., Baudoin, J.P., Mergeai, G., 2009. Expression of the “glanded-plant and glandless-seed” trait of Australian diploid cottons in different genetic backgrounds. Euphytica 165 (2), 211–221. https://doi.org/10.1007/s10681-008-9772-8.

Butt, T.M., Goettel, M.S., 2000. In: Bioassays of entomopathogenic microbes and nematodes.. CABI, Wallingford, pp. 141–195. https://doi.org/10.1079/9780851994222.0141.

Butt, T.M., Wang, C., Shah, F.A., Hall, R., 2006. In: Progress in Biological ControlAn Ecological and Societal Approach to Biological Control. Springer Netherlands, Dordrecht, pp. 213–226. https://doi.org/10.1007/978-1-4020-4401-4_10.

Cherry, A.J., Banito, A., Djegui, D., Lomer, C., 2004. Suppression of the stem-borer Sesamia calamistis (Lepidoptera; Noctuidae) in maize following seed dressing, topical application and stem injection with African isolates of Beauveria bassiana. Int. J. Pest Manag. 50 (1), 67–73. https://doi.org/10.1080/09670870310001637426.

Dutky, S.R., Thompson, J.V., Cantwell, G.E., 1962. A technique for mass rearing the greater wax moth. Proc. Entomol. Soc. Wash 64, 56–58.

Egbuta, M.A., McIntosh, S., Waters, D.L.E., Vancov, T., Liu, L., 2017. Biological importance of cotton by-products relative to chemical constituents of the cotton plant. Molecules 22, 12–14. https://doi.org/10.3390/molecules22010093.

Fuchs, B.B., O’Brien, E., El Khoury, J.B., Mylonakis, E., 2010. Methods for using Galleria mellonella as a model host to study fungal pathogenesis. Virulence 1 (6), 475–482. https://doi.org/10.4161/viru.1.6.12985.

Gao, Q., Jin, K., Ying, S.-H., Zhang, Y., Xiao, G., Shang, Y., Duan, Z., Hu, X., Xie, X.-Q., Zhou, G., Peng, G., Luo, Z., Huang, W., Wang, B., Fang, W., Wang, S., Zhong, Y.i., Ma, L.-J., St. Leger, R.J., Zhao, G.-P., Pei, Y., Feng, M.-G., Xia, Y., Wang, C., Achtman, M., 2011. Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi Metarhizium anisopliae and M. acridum. PLoS Genet. 7 (1), e1001264. https://doi.org/10.1371/journal.pgen.1001264.

Garrido-Jurado, I., Resquín-Romero, G., Amarilla, S.P., Ríos-Moreno, A., Carrasco, L., Quesada-Moraga, E., 2017. Transient endophytic colonization of melon plants by entomopathogenic fungi after foliar application for the control of Bemisia tabaci Gennadius (Hemiptera: Aleyrodidae). J. Pest Sci. (2004). 90, 319–330. 10.1007/s10340-016-0767-2.

González-Mas, N., Sánchez-Ortiz, A., Valverde-García, P., Quesada-Moraga, E., 2019. Effects of endophytic entomopathogenic ascomycetes on the life-history traits of Aphis gossypii Glover. Insects 10, 165. https://doi.org/10.3390/insects10060165.

Gurulingappa, P., Sword, G.A., Murdoch, G., McGee, P.A., 2010. Colonization of crop plants by fungal entomopathogens and their effects on two insect pests when in planta. Biol. Control 55 (1), 34–41. https://doi.org/10.1016/j.biocontrol.2010.06.011.

Hu, S., Bidochka, M.J., 2021. Root colonization by endophytic insect-pathogenic fungi. J. Appl. Microbiol. 130 (2), 570–581. https://doi.org/10.1111/jam.v130.210.1111/jam.14503.

Jaber, L.R., Vidal, S., 2010. Fungal endophyte negative effects on herbivory are enhanced on intact plants and maintained in a subsequent generation. Ecol. Entomol. 35, 25–36. https://doi.org/10.1111/j.1365-2311.2009.01152.x.

Kaplan, E.L., Meier, P., 1958. Nonparametric Estimation from Incomplete Observations. J. Am. Stat. Assoc. 53 (282), 457–481. https://doi.org/10.1080/01621459.1958.10501452.

Keyser, C.A., Thorup-Kristensen, K., Meyling, N.V., 2014. Metarhizium seed treatment mediates fungal dispersal via roots and induces infections in insects. Fungal Ecol. 11, 122–131. https://doi.org/10.1016/j.funeco.2014.05.005.

Klieber, J., Reineke, A., 2016. The entomopathogen Beauveria bassiana has epiphytic and endophytic activity against the tomato leaf miner Tuta absoluta. J. Appl. Entomol. 140 (8), 580–589. https://doi.org/10.1111/jen.12287.

Loesch, A., Hutwimmer, S., Strasser, H., 2010. Carbon utilization pattern as a potential quality control criterion for virulence of Beauveria brongniartii. J. Invertebr. Pathol. 104 (1), 58–65. https://doi.org/10.1016/j.jip.2010.01.007.

Lopez, D.C., Sword, G.A., 2015. The endophytic fungal entomopathogens Beauveria bassiana and Purpureocillium lilacinum enhance the growth of cultivated cotton (Gossypium hirsutum) and negatively affect survival of the cotton bollworm (Helicoverpa zea). Biol. Control 89, 53–60. https://doi.org/10.1016/j.biocontrol.2015.03.010.

Lovett, B., St. Leger, R.J., 2017. The insect pathogens. Microbiol. Spectr. 5, 923–943. https://doi.org/10.1128/9781555819583.ch45.

Mellon, J.E., Zelaya, C.A., Dowd, M.K., Beltz, S.B., Klich, M.A., 2012. Inhibitory effects of gossypol, gossypolone, and apogossypolone on a collection of economically important filamentous fungi. J. Agric. Food Chem. 60 (10), 2740–2745. https://doi.org/10.1021/jf2044394.

Meyling, N.V., Eilenberg, Jørgen, 2006. Isolation and characterisation of Beauveria bassiana isolates from phylloplanes of hedgerow vegetation. Mycol. Res. 110 (2), 188–195. https://doi.org/10.1016/j.mycres.2005.09.008.

Moonjely, S., Bidochka, M.J., 2019. Generalist and specialist Metarhizium insect pathogens retain ancestral ability to colonize plant roots. Fungal Ecol. 41, 209–217. https://doi.org/10.1016/j.funeco.2019.06.004.

Poprawski, T.J., Jones, W.J., 2000. Host plant effects on activity of the mitosporic fungi Beauveria bassiana and Paecilomyces fumosoroseus against two populations of Bemisia whiteflies (Homoptera: Aleyrodidae). Mycopathologia 151, 11–20. https://doi.org/10.1023/A:1010835224472.

Powell, W.A., Klingeman, W.E., Ownley, B.H., Gwinn, K.D., 2009. Evidence of endophytic Beauveria bassiana in seed-treated tomato plants acting as a systemic entomopathogen to larval Helicoverpa zea (Lepidoptera: Noctuidae). J. Entomol. Sci. 44, 391–396. https://doi.org/10.18474/0749-8004-44.4.391.

Powell, W.A., Klingeman, W.E., Ownley, B.H., Gwinn, K.D., Dee, M., Flanagan, P.C., 2007. Endophytic Beauveria bassiana in tomatoes yields mycosis in tomato fruitworm larvae. HortScience 42, 933.

Przybylski, P., Pyta, K., Remlein-Starosta, D., Schroeder, G., Brzezinski, B., Bartl, F., 2009. Antifungal activity of alkyl and heterocyclic aza-derivatives of gossypol as well as their complexes with NaClO4 against Fusarium oxysporum f. sp. lupini.

Bioorganic Med. Chem. Lett. 19 (7), 1996–2000. https://doi.org/10.1016/j.bmcl.2009.02.051.

Quesada Moraga, E., 2020. Entomopathogenic fungi as endophytes: their broader contribution to IPM and crop production. Biocontrol Sci. Technol. 30 (9), 864–877. https://doi.org/10.1080/09583157.2020.1771279.

Quesada-Moraga, E., Muñoz-Ledesma, F.J., Santiago-Álvarez, C., 2009. Systemic protection of Papaver somniferum L. against Iraella luteipes (Hymenoptera: Cynipidae) by an endophytic strain of Beauveria bassiana (Ascomycota: Hypocreales). Environ. Entomol. 38 (3), 723–730. https://doi.org/10.1603/022.038.0324.

Quesada-Moraga, E., Vey, A., 2003. Intra-specific variation in virulence and in vitro production of macromolecular toxins active against locust among Beauveria bassiana strains and effects of in vivo and in vitro passage on these factors. Biocontrol Sci. Technol. 13 (3), 323–340. https://doi.org/10.1080/0958315031000110346.

Resquín-Romero, G., Garrido-Jurado, I., Delso, C., Ríos-Moreno, A., Quesada-Moraga, E., 2016. Transient endophytic colonizations of plants improve the outcome of foliar applications of mycoinsecticides against chewing insects. J. Invertebr. Pathol. 136, 23–31. https://doi.org/10.1016/j.jip.2016.03.003.

Rivas-Franco, F., Hampton, J.G., Narciso, J., Rostás, M., Wessman, P., Saville, D.J., Jackson, T.A., Glare, T.R., 2020. Effects of a maize root pest and fungal pathogen on entomopathogenic fungal rhizosphere colonization, endophytism and induction of plant hormones. Biol. Control 150, 104347. https://doi.org/10.1016/j.biocontrol.2020.104347.

Sánchez-Rodríguez, A.R., Raya-Díaz, S., Zamarreño, Á.M., García-Mina, J.M., del Campillo, M.C., Quesada-Moraga, E., 2018. An endophytic Beauveria bassiana strain increases spike production in bread and durum wheat plants and effectively controls cotton leafworm (Spodoptera littoralis) larvae. Biol. Control 116, 90–102. https://doi.org/10.1016/j.biocontrol.2017.01.012.

Santiago-Álvarez, Cándido, Maranhão, E.A., Maranhão, E., Quesada-Moraga, E., 2006. Host plant influences pathogenicity of Beauveria bassiana to Bemisia tabaci and its sporulation on cadavers. BioControl 51 (4), 519–532. https://doi.org/10.1007/s10526-005-5737-1.

Screen, S.E., St. Leger, R.J., 2000. Cloning, expression, and substrate specificity of a fungal chymotrypsin. Evidence for lateral gene transfer from an actinomycete bacterium. J. Biol. Chem. 275, 6689–6694. https://doi.org/10.1074/jbc.275.9.6689.

Shah, F.A., Butt, T.M., 2005. Influence of nutrition on the production and physiology of sectors produced by the insect pathogenic fungus Metarhizium anisopliae. FEMS Microbiol. Lett. 250, 201–207. https://doi.org/10.1016/j.femsle.2005.07.011.

Steel, R., Torrie, J.H., 1985. Bioestadistica principios y procedimientos, 2nd, editio. ed. McGraw-Hill, Bogotá.

Stone, L.B.L., Bidochka, M.J., 2020. The multifunctional lifestyles of Metarhizium: evolution and applications. Appl. Microbiol. Biotechnol. 104 (23), 9935–9945. https://doi.org/10.1007/s00253-020-10968-3.

Vega, F.E., 2018. The use of fungal entomopathogens as endophytes in biological control: a review. Mycologia 110, 4–30. https://doi.org/10.1080/00275514.2017.1418578.

Vega, F.E., Posada, F., Catherine Aime, M., Pava-Ripoll, M., Infante, F., Rehner, S.A., 2008. Entomopathogenic fungal endophytes. Biol. Control 46 (1), 72–82. https://doi.org/10.1016/j.biocontrol.2008.01.008.

Zhang, Y., Zhang, J., Jiang, X., Wang, G., Luo, Z., Fan, Y., Wu, Z., Pei, Y., 2010. Requirement of a mitogen-activated protein kinase for appressorium formation and penetration of insect cuticle by the entomopathogenic fungus Beauveria bassiana. Appl. Environ. Microbiol. 76 (7), 2262–2270. https://doi.org/10.1128/AEM.02246-09.